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Carbonic anhydrase activity as a potential biomarker for acute exposure to copper in corals

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HIGHLIGHTS

- Cu exposure may affect the calcification process of corals.
- The Cu affects on the CA activity through different experimental setups.
- Mesocosm systems can be a more realistic and reliable experimental approach.
- A reduction in CA activity was observed in the coral *M. harttii* exposed to Cu.
- CA activity proved to be a potential biomarker sensitive to Cu.

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ABSTRACT

Coral reefs are subjected to climate change and are severely impacted by human activities, with copper (Cu) being a relevant physiological stressor for corals at local scale. The ecological relevance of parameters measured at biochemical or cellular level is now considered an extremely important feature in environmental studies, and can be used as early warning signs of environmental degradation. In this context, the effects of acute exposure (96 h) to Cu were assessed on the maximum photochemical efficiency of zooxanthellae (*Fv/Fm*) and on the activity of key enzymes [carbonic anhydrase (CA) and Ca-ATPase] involved in coral physiology using the scleractinian coral *Mussismilia harttii* as a biological model. Corals were exposed to different concentrations of dissolved Cu (4.6–19.4 μg/L) using two different experimental approaches: a laboratory closed system and a marine mesocosm system. *Fv/Fm* values and Ca - ATPase activity were not affect by exposure to Cu in any of the exposure systems. However, a significant reduction in CA activity was observed in corals exposed to 11.9 and 19.4 μg Cu/L in the laboratory and at all concentrations of Cu tested in the mesocosm system (4.6, 6.0 and 8.5 μg/L). Based on the sensitivity of this enzyme to the short period of exposure to sublethal concentrations of Cu in both experimental approaches, the present study suggests the use of CA activity as a potential biomarker to be used in biomarker-based environmental monitoring programs in coral reefs.

1. Introduction

Aquatic ecosystems are among the main final receptacles for point and diffuse contamination (Duda, 1993; Muller et al., 2002). Rivers and streams contaminated by agricultural, urban and industrial activities are generally the most important routes for entry of contaminants into marine environments (van Dam et al., 2011).

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Among the contaminants occurring in aquatic ecosystems, metals such as copper (Cu), can be introduced by several sources, such as industrial, domestic and agricultural wastes, fossil fuel burning and antifouling paints (Maria and Bebianno, 2011). Cu is an essential metal for physiological functions (Morgan, 2000) acting as an enzymatic cofactor (Rubino and Franz, 2012), however, it can induce adverse effects in organisms at excessive concentrations (Liu et al., 2006).

Among marine ecosystems, coral reefs stand out due to their high productivity and biological diversity (Connell, 1978; Bertucci et al., 2013). The formation of coral reefs is highly dependent on the mutualistic relationship between scleractinian corals and dinoflagellates from the family Symbiodiniaceae, known as zooxanthellae (Hoegh-Guldberg, 1999; LaJeunesse et al., 2018). This symbiosis contributes to the calcification process performed by corals, essential to the foundation of reef structure (Bertucci et al., 2013). Nearshore coral reefs are more likely exposed to stressors (Fabricius, 2005; Leão and Kikuchi, 2005; Smith et al., 2008), and contamination with metals, such as Cu, is a notable local impact threatening coral reefs in many locations worldwide (van Dam et al., 2011). The exposure of corals to excessive concentrations of Cu may trigger metabolic and oxidative stress (Nystrom et al., 2001; Schwarz et al., 2013; Bielmyer-Fraser et al., 2018), bleaching (Grant et al., 2003; Yost et al., 2010), and impairment in the calcification process (Bielmyer et al., 2010; Fonseca et al., 2017; Marangoni et al., 2017b). Also, it is important to note that toxicity of metals, including Cu, is expected to be augmented in the context of climate change (Nikinmaa, 2013).

Biological effects have been increasingly used as a way to assess corals heath impairment caused by chemical contamination and variations in environmental conditions (Edge et al., 2005; Rodriguez-Lanetty et al., 2009; Bielmyer et al., 2010; Bielmyer-Fraser et al., 2018). Gene expression (Venn et al., 2009, Rocker et al., 2015), enzymatic activity (Bielmyer et al., 2010; Fonseca et al., 2017; Marangoni et al., 2017a, b; Bielmyer-Fraser et al., 2018), photosynthetic efficiency (Owen et al., 2002; Jones, 2005; Cantin et al., 2007; Fonseca et al., 2017; Marangoni et al., 2019) and −"omics" based approaches (ReFuGe 2020 Consortium, 2015) are some of the parameters that can be listed as potential biological markers (or biomarkers) to assess corals health.

Biomarkers, which can be defined as any biological response in molecular, cellular and physiological levels, are useful to indentify adverse effects caused by environmental stressors in marine organisms (McCarthy and Shugart, 1990; Bradley, 2012). Based on an understanding of cellular-level processes, biochemical responses can predict biological effects linked to environmental degradation (Depledge et al., 1995; Downs et al., 2005). In this context, it is worth noting that biochemical techniques can be more inexpensive and practical compared to other techniques (e.g. molecular −omics techniches), and capable of diagnosing the impact of stressors under controlled conditions (Carvalho and Fernandes, 2008; Oliveira et al., 2018) or *in situ* (Prazeres et al., 2012; Marques et al., 2019). This are desirable features for biomarkers used in large-scale environmental monitoring programs, since their favor reproducibility (Hagger et al., 2006). Also, given that corals are metaorganisms comprised of the coral animal host, its intracellular photosynthetic algae, and associated microbiota, it is important that efforts on strategies for mitigating future reef loss focus on entire coral holobionts responses to stressors (ReFuGe 2020 Consortium, 2015).

Coral bleaching, characterized by the loss of zooxanthellae or reduction of the photosynthetic pigments of these microalgae (Downs et al., 2002), can severely affect corals health as they are deprived of organic nutrients coming from photosynthesis (Ferrier-Pagès et al., 1998; Colombo-Pallotta et al., 2010). Within this context, fluorescence technology of chlorophyll *a* allows to quickly assess the photosynthetic efficiency of zooxanthellae *(Fv/Fm)*, and such technique has been extensively used to address the health and integrity of the important symbiotic relationship established between corals and dinoflagellates (e.g. Jones et al., 1999; Silverstein et al., 2014).

In turn, the calcification process performed by some reef organisms is a physiological characteristic that can indicate how healthy the reef is and how it is growing (Hoegh-Guldberg, 1999; De'ath et al., 2009; Weis and Allemand, 2009). Biochemical measurements, such as activity of enzymes, related to this process can be used to assess corals health status before significant damages occur (Prazeres et al., 2015; Fonseca et al., 2017; Marangoni et al., 2017 ab; Marques et al., 2017). Specifically, carbonic anhydrase (CA) and Ca^{2+}- activated, Mg^{2+-}dependent ATPase (hereafter referred to as Ca-ATPase) are considered key enzymes in this process. The first catalyzes the reversible hydration reaction of carbon dioxide (CO_2) in bicarbonate (HCO_3^-) and plays a key role not only in the supply of dissolved inorganic carbon (DIC) for calcification but also for photosynthesis (Bertucci et al., 2013). In turn, Ca-ATPase transport Ca^{2+} into the calcification site while concomitantly removes protons (H^+) from this medium (Rollion-Bard and Blamart, 2015), directing the calcification reaction toward the formation of $CaCO_3$ (Al-Horani et al., 2003; Cohen and McConnaughey, 2003; Allemand et al., 2011).

Considering that coral reefs are severely impacted by human activities and subject to climate change (Hughes et al., 2003; Pandolfi et al., 2011; van Dam et al., 2011; Hughes et al., 2017, 2018), the use of biochemical biomarkers as complementary tools for coral health assessment can be extremely beneficial in reef monitoring programs (Downs et al., 2005; Bradley, 2012). Also, acute experiments (96 h exposure) are performed in the ecotoxicology field as an effective way to detect stress response mechanisms in organisms (van Dam et al., 2011), and can be also useful for the detection of potential biomarkers.

Here we aimed to evaluate maximum photochemical efficiency of zooxanthellae (*Fv/Fm*) and the response of key enzymes (CA and Ca-ATPase) involved in the photosynthetic and calcification processes of scleractinian corals as potential biomarkers for acute exposure to Cu. The coral holobiont *Mussismilia harttii,* an important reef builder in South Atlantic reefs (Castro and Pires, 2001), was used as a biological model. Corals were maintained under control condition and exposed to increasing Cu concentrations in two different experimental setups contemplating controlled laboratory conditions and a flow-throw outdoor mesocosm system.

2. Materials and methods

2.1. Organisms collection

M. harttii fragments coming from 3 different colonies were collected by scuba diving in the conservation area of the Municipal Natural Park of Recife de Fora (MNPRF), Porto Seguro, BA, northeastern Brazil (16°25′08.1″S/38°58′54.1″W), in July 2012. Coral fragments were transported to the experimental facilities of the Coral Vivo Project (Arraial d'Ajuda, Porto Seguro, BA, Brazil). Collection was conducted under the permission of the Brazilian Environmental Agency (IBAMA/SISBIO; permit # 34256-1). Polyps were individualized, glued on ceramic plates using cyanoacrylate (Fig. 1), and acclimated for two weeks in laboratory and mesocosm conditions before Cu exposure.

2.2. Laboratory and mesocosm acute exposure to copper

Corals were exposed in laboratory conditions to four different

Fig. 1. Picture of polyps of *Mussismilia harttii* individualized and glued on ceramic plates.

nominal concentrations of Cu (0, 5, 9 and 20 μg Cu/L) for 96 h. The concentrations of Cu tested were based on international and Brazilian legislations for the protection of aquatic environments (CONAMA, 2005; EPA, 2005), being the lower concentrations representative of the quality criteria for marine (5 μg/L) and fresh water (9 μg/L) environments, while the highest one (20 μg/L) represents a extrapolation of the limits stipulated by the legislation, a "non-conforming" condition. Corals were kept in a closed system of 12 aquariums (30 L) containing seawater collected in the Araçaípe Beach reef (Arraial d'Ajuda, Bahia, northeastern of Brazil) with constant aeration, daily renewal of the experimental medium and artificial light set up to ~120 μmol photons $m^{-2}s^{-1}$ (12:12 photoperiod).

In the marine mesocosm corals were exposed to four different environmentally relevant concentrations of Cu (nominal concentrations of 0,1, 3, 5 μg/L) for 96 h using a secondary system especially built for ecotoxicological studies (see Duarte et al., 2015). A flow-through system with 12 aquariums (10 L) containing seawater pumped from the reef environment (Araçaípe Beach reef) was used to conduct the experiment. Lower Cu concentrations were tested in the mesocosm system aiming a more ecologically realistic approach. Stock Cu solutions with final nominal concentrations of 10, 30 and 50 μg/L were prepared daily in 1000-L tanks containing seawater pumped from the coral reef area. Considering that 10% of the flow reaching the test aquaria came from these tanks with stock solutions, measured concentrations of Cu in the test aquariums were expected to be 0, 1, 3 and 5 μg/L. The experimental conditions (seawater composition, temperature, pH, turbidity, salinity, plankton density, photoperiod, rainfall, light incidence, among others) were similar to the natural environment since due to the mesocosm direct connection and permeability to the reef environment (Duarte et al., 2015). Natural light was attenuated by Sombrite®Fabric 70% reaching an irradiance intensity (250 μmol photons $m^{-1}s^{-1}$) equivalent to the maximum value observed at 2.5 m depth (noontime) in the site of coral collection. Both experiments were performed in triplicate (3 aquaria per treatment). Cu stock solutions were prepared daily, from a stock solution of $CuCl_2$ (1 g/LCu), 24 h prior to exposure to allow the total balance of Cu in seawater (Lauer et al., 2012; Machado et al., 2013).

Coral polyps were collected one day before the beginning of the experiment (acclimated corals) and after 96 h of exposure (n = 9 per treatment in laboratory conditions; n = 3 per treatment in the mesocosm). After collection corals were immediately stored in liquid nitrogen for further analyses of biochemical parameters. Data of acclimated corals and control corals after 96 h exposure were compared to assess: (i) the adequacy of acclimation conditions used, (ii) the overall fitness of control corals over the experiment, (iii) and possible influences of natural shifts in physicochemical parameters in the mesocosm system in biomarkers responses.

2.3. Concentration of dissolved copper in water

Water samples from each treatment were collected every 24 h over the experimental period (96 h). Filtered (0.45-μm mesh filters) samples were stored in 15-mL Falcon-type tubes and acidified with HNO_3 (1% final concentration; SupraPur, Merck, Darmstadt, Germany). Samples were desalted as described by Nadella et al. (2009) and Cu concentrations were analyzed by atomic absorption spectrophotometry with coupled graphite furnace (Perkin-Elmer, Waltham, MA, USA).

2.4. Maximum photosynthetic capacity of photosystem II (Fv/Fm)

Symbiont maximum photochemical efficiency of photosystem II *(Fv/Fm)* was measured using a pulse-amplitude modulated fluorometer (Diving-PAM, Walz, Germany). Measurements were performed as described by Marangoni et al. (2017b) using dark-acclimated (30 min) corals randomly selected. *Fv/Fm* values represent an estimate of zooxanthellae health, with decreased ratios indicating loss in the efficiency of photochemical energy conversion and or damage in the PS-II (Jones, 2005).

2.5. Activity of carbonic anhydrase (CA) and Ca-ATPase

Holobiont sample preparation for enzyme activity analyses was performed following procedures described by Downs et al. (2005), with some modifications reported by Marangoni et al. (2017b). Briefly, samples were macerated in liquid nitrogen and sonicated (70 kHz, Sonaer Ultrasonics, NY, USA) in ice using the specific homogenization buffer (1:2, wv) required for each enzyme assay. After homogenization, samples were centrifuged (13,000 g, 10 min, 4 °C) and supernatant was used as enzyme source. The total protein content in the sample homogenate was determined using a commercial reagent kit based on the Bradford assay (Sigma-Aldrich, St. Louis, MO, USA).

CA activity measurement was based on the method described by Henry (1991). The buffer used for samples sonication had the following composition: Tris-Base (10 mM, pH 8.5), sucrose (75 mM), protease inhibitor (1 mM phenylmethanesulfonyl-PMSF fluoride) and ditritiotreitol (1 mM DTT). Homogenized sample was added to a reaction solution composed of Tris-Base (10 mM, pH 8.5), sucrose (75 mM), mannitol (225 mM) and phosphate (10 mM). Subsequently, substrate (obtained by saturating ultrapure water with CO_2) was added and the pH was recorded (ambient temperature approximately 24 °C). Blank measurements were carried out simultaneously by replacing the sample homogenate by same buffer solution used for sample homogenization. A linear regression model (dependent variable: pH, independent variable: time) was used to determine the slope of the reaction. Values obtained from the homogenates represents the catalyzed reaction rate, while values obtained for blank measurements represent the rate of the non-catalyzed reaction. Data were expressed as unit of CA/mg protein.

Ca-ATPase activity was determined based on the protocol described by Vajreswari et al. (1983), with some modifications. Samples were homogenized in a buffer containing Tris-HCl (100 mM, pH 7.6), sucrose (500 mM), DTT (1 mM) and PMSF (1 mM). The reaction medium used was composed of NaCl (189 mM), $MgCl_2$ (5 mM), $CaCl_2$ (5 mM), ouabain (1 mM), Tris-HCl (20 mM, pH 7.6) and ATP (3 mM). The concentration of inorganic phosphate (Pi) released by the activity of the enzymes in the reaction medium was determined using the commercial reagent kit "Fosfato" (Doles Reagentes, Goiânia, GO, Brazil). Sample absorbance was measured at 630 nm. Data were expressed in mmol Pi/mg protein/min.

2.6. Statistical analysis

Data were expressed as mean ± standard error. The effects of the different concentrations of Cu on the *Fv/Fm* values and enzymatic activities were compared using one-way analysis of variance ANOVA, followed by the Fisher test for multiple comparisons. Data normality and homogeneity of variances were previously verified using the Shapiro-Wilk and Cochran C tests, respectively. The confidence level adopted was 95% ($\alpha = 0.05$).

3. Results

3.1. Water physicochemical parameters

Seawater physicochemical parameters were similar throughout experiments. Laboratory conditions of dissolved oxygen, temperature, salinity and pH corresponded to 7.26 ± 0.05 mg/L, 23.6 ± 0.1 °C, 37.2 ± 0.1 ppt and 8.1 ± 0.01, respectively. Mean values of dissolved Cu concentrations in the experimental media were 2.9 ± 0.6, 9.2 ± 2.2, 11.9 ± 3.2, 19.4 ± 5.0 μg Cu/L, for nominal concentrations of 0 (control, without addition of Cu in seawater), 5, 9 and 20 μg Cu/L. For the mesocosm, dissolved oxygen, temperature, salinity, pH and pluviometry corresponded to 5.35 ± 0.35 mg/L, 25.2 ± 0.05 °C, 35.5 ± 0.06 ppt, 8.29 ± 0.00 and 0.23 ± 0.05 mm, respectively. Mean values of the dissolved Cu concentration were 3.3 ± 0.6, 4.6 ± 0.5, 6.0 ± 0.9, and 8.5 ± 1.0 μg Cu/L, for the nominal concentrations of 0 (control, without addition of Cu in sea water), 1, 3 and 5 μg Cu/L.

3.2. Acclimated corals versus control group

No significant differences were observed for *Fv/Fm* values or for CA and Ca- ATPase activity between acclimated corals and the control group maintained for 96 h in the laboratory and mesocosm systems (Table 1). Such results indicate a steady state of corals during experiments, reinforcing that possible effects observed in the physiological parameters evaluated in *M. harttii* can be attributed to Cu toxicity.

3.3. Maximum photosynthetic capacity of photosystem II (Fv/Fm)

No changes were observed in *Fv/Fm* values in corals maintained under control condition compared to those exposed for 96 h at different concentrations of Cu in both laboratory ($p = 0.47$) (Fig. 2A) and mesocosm experiments ($p = 0.38$) (Fig. 2B).

3.4. Enzymatic activities

After 96 h of exposure in laboratory, CA activity was significantly reduced in corals exposed to 11.9 and 19.4 μg/L of Cu compared to control condition ($p \leq 0.03$). Corals exposed to 11.9 μg Cu/L also showed a significant reduction in CA activity compared to corals exposed to 9.2 μg/L ($p = 0.036$, Fig. 3A). In turn, corals exposed at the mesocosm system, presented a significant CA inhibition at all tested Cu concentrations, compared to corals under control condition ($p \leq 0.04$) (Fig. 3B).

Regarding Ca-ATPase, higher enzyme activity was observed in corals exposed to 11.9 μg/L Cu compared to those exposed to 9.2 μg/L Cu in laboratory experiment ($p = 0.04$ Fig. 4A). However, in comparison to control conditions, no significant changes was observed for corals exposed to Cu in both laboratory ($p = 0.24$, Fig. 4A) and mesocosm ($p = 0.78$, Fig. 4B) experiments. Statistical results are presented in Table 2.

4. Discussion

Metals have become one of the main pollutants in the marine environment and a major threat to the growth and reproduction of stony corals. Specifically, Cu pollution can be a relevant issue in coral reefs (van Dam et al., 2011). By using two different experimental approaches, and evaluating ecologically relevant physiological endpoints in the coral *M. harttii*, the present study was able to detect CA activity as a sensitive biochemical response to

Table 1
Mean and standard error of parameters evaluated (*Fv/Fm* and activity of CA and Ca-ATPase) in the coral *Mussismilia hartti* acclimated for two weeks and maintained in the control group for 96 h in the laboratory and mesocosm.

Evaluated parameters	Acclimated	Control	*p*
Fv/Fm in laboratory	622.8 ± 16.77	623 ± 12.74	0.993
Fv/Fm in mesosm	619.3 ± 25.98	618 ± 13.93	0.983
CA activity in laboratory	190.4 ± 30.3	200.6 ± 24.9	0.777
CA activity in mesocosm	283.9 ± 66.4	453.5 ± 41.48	0.561
Ca-ATPase activity in laboratory	3.79 ± 0.63	3.37 ± 0.45	0.598
Ca-ATPase activity in mesocosm	2.85 ± 0.66	3.41 ± 0.36	0.254

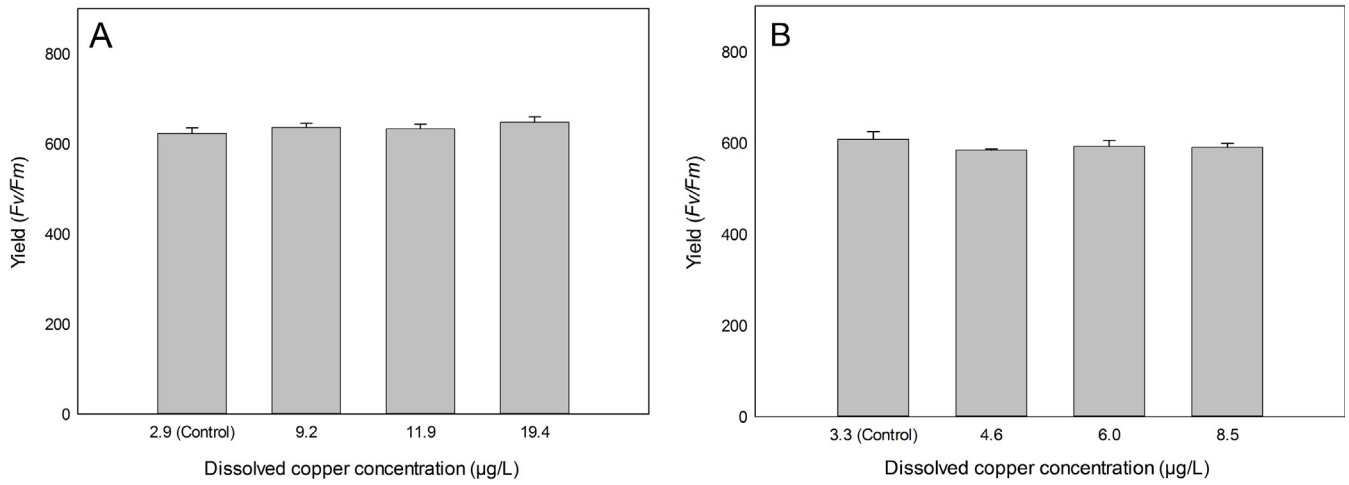

Fig. 2. Photochemical efficiency of the photosystem II (*Fv/Fm*) of symbiotic algae in the coral *Mussismilia harttii* exposed to control condition (no Cu addition in seawater) or exposed to increasing dissolved Cu concentrations in seawater for 96 h in laboratory (A) and mesocosm system (B). Data are expressed as mean ± standard error.

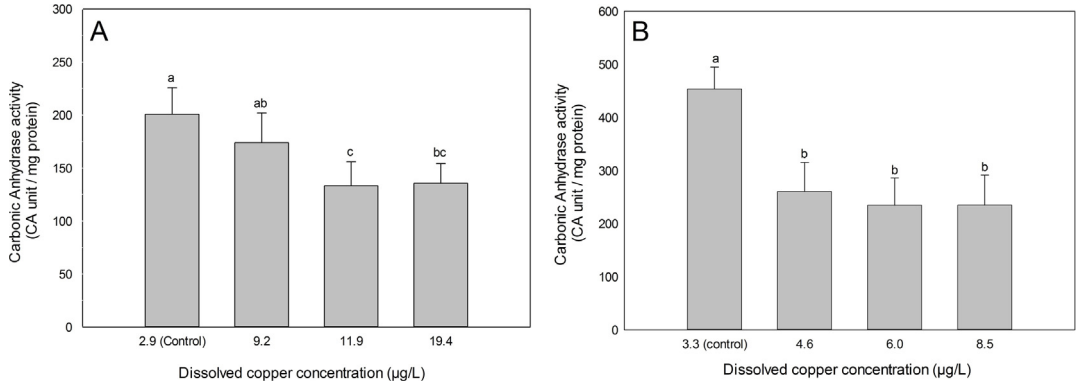

Fig. 3. Carbonic anhydrase (CA) activity in the coral *Mussismilia harttii* exposed to control condition (no Cu addition in seawater) or exposed to increasing dissolved Cu concentrations in seawater for 96 h in laboratory (A) and mesocosm system (B). Data are expressed as mean ± standard error. Different letters indicate significantly different mean values ($p < 0.05$) among treatments.

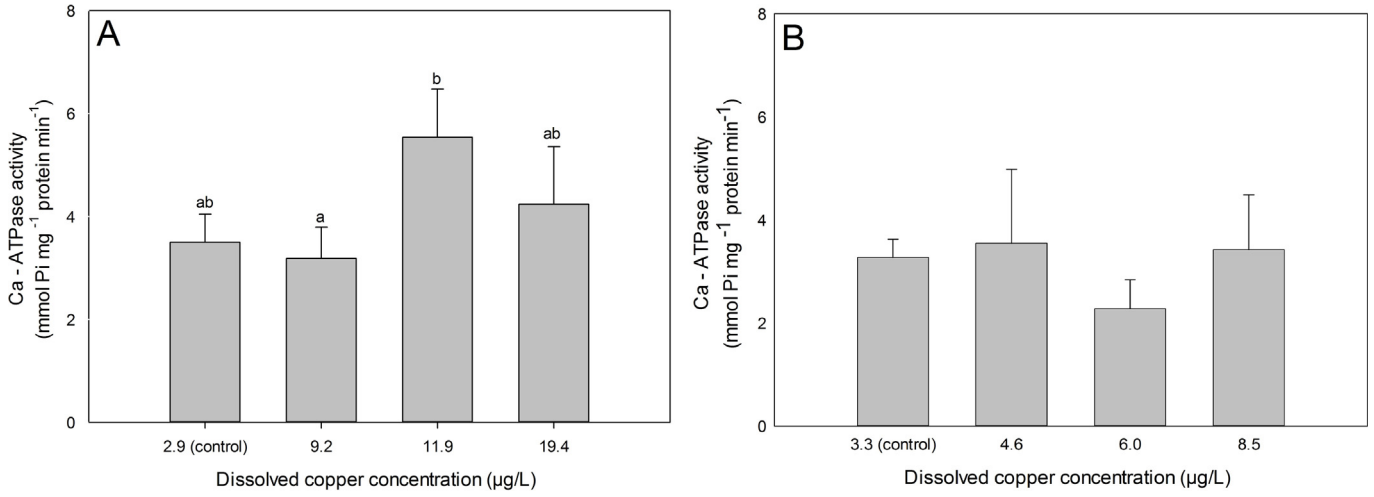

Fig. 4. Ca- ATPase activity in the coral *Mussismilia harttii* exposed to control condition (no Cu addition in seawater) or exposed to increasing dissolved Cu concentrations in seawater for 96 h in laboratory (A) and mesocosm system (B). Data are expressed as mean ± standard error. Different letters indicate significantly different mean values ($p < 0.05$) among treatments.

ecological relevant concentrations of dissolved Cu in seawater, which suggests CA activity as a potential biomarker to early detect the possible deleterious effects of Cu in corals health.

Despite the fact that Cu, as well as other metals, can replace the

Table 2
Results of ANOVA of the parameters evaluated (*Fv/Fm* and activity of CA and Ca ATPase) in *Mussismilia harttii* exposed to different concentrations of dissolved Cu in seawater through different experimental setups: laboratory and marine mesocosm for 96 h. Significant values ($p < 0.05$) are in bold.

Evaluated parameters	*DF*	*F*	*p*
Fv/Fm in laboratory	3	0.8633	0.4701
Fv/Fm in mesocosm	3	1.153	0.3855
CA activity in laboratory	3	3.387	**0.0312**
CA activity in mesocosm	3	4.205	**0.0462**
Ca-ATPase activity in laboratory	4	1.422	0.2440
Ca- ATPase activity in mesocosm	3	0.3652	0.7800

magnesium in chlorophyll *a* molecules causing deleterious effects to the photosynthetic process (Kupper et al., 1996), no effects on *Fv/Fm* values were observed in the present study. Marangoni et al. (2017b) did not observe changes in *Fv/Fm* values for *M. harttii* exposed to Cu concentrations (up to 6.7 μg/L) for 12 days, however, in a more recent study (Marangoni et al., 2019), such specie showed a decreasing in *FvFm* values after 35 days of exposure to 3.2 μg/L of Cu. Bielmyer et al. (2010) showed a significant reduction on *Fv/Fm* in the coral *Pocillopora damicornis* exposed to 4 μg/L of Cu, while no effects were observed in *Acropora cervicornis* and *Montastraea faveolata* exposed to higher Cu concentrations (up to 10 μg/L) after 35 days of exposure.

Coral species-specific sensitivity to Cu are partly driven by the different algal symbiont communities (Bielmyer et al., 2010), especially considering deleterious effects to the photosynthetic metabolism. Also, according to Kuzminov et al. (2013) the toxic effects of Cu in symbiotic dinoflagellates (*Symbiodinium microadriaticum*) isolated from corals has as primary target processes downstream PSII rather than photochemical process in PSII. Considering the short-period exposure (96 h) used in the present study, this could be a possible physiological explanation for the lack effect observed even when higher Cu concentration were tested (up to 19.4 μg/L). The present results and previous reports suggest *FvFm* value as a potential biomarker to identify health impairment in corals after chronic exposure to low Cu concentrations. Identification of such biomarkers is also extremely relevant for monitoring purposes; however, this is beyond the scope of the present study that aimed to detect acute exposure biomarkers.

Metals can also inhibit ATPases by binding to sulfhydryl groups (-SH) consequently changing the structure of proteins (Viarengo et al., 1996; Dias and Coelho, 2007; Jin et al., 2015). Changes in the Ca^{-}ATPase activity have been shown to be related to calcification rates in reef organisms (Prazeres et al., 2015; Marangoni et al., 2017a), however, no effects due to Cu exposure on the activity of Ca^{-}ATPase were here observed. Marangoni et al. (2017b) reported Cu exposure effects on the activity of Ca^{-}ATPase in *M. harttii*, however, decreased enzyme activity was detected in a period of exposure three times longer than in the present experiment. In addition, Fonseca et al. (2017) also observed changes on Ca-ATPase activity in *M. harttii* after 8 days of exposure to increasing Cu concentrations (up to 8.6 μg/L) combined with increasing temperature. Information on metal effects in Ca-ATPase activity concerning other coral species are lacking. Nevertheless, results from the present and previous studies using *M. harttii* as a biological model suggest that Ca^{-}ATPase may present a later response regarding Cu toxicity in corals.

Concerning CA, an activity inhibition of approximately 35% and 45% due to Cu exposure were observed in both laboratory and mesocosm experiments, respectively. Previous investigations also reported the same effect in CA activity due to Cu exposure in marine invertebrates, including corals. Gilbert and Guzman (2001) observed CA activity inhibition in two species of anemones (*Condylactis gigantea* and *Stichodactyla helianthus*) exposed for 48 h at Cu concentrations of 10 and 40 μg/L. Bielmyer et al. (2010) observed a decreased activity of this enzyme in the corals *A. cervicornis* and *M. faveolata* exposed to 10 and 20 μg Cu/L for 5 weeks. In turn, Marangoni et al. (2019) observed CA activity inhibition in *M. harttii* at Cu concentrations as low as 2.3 μg/L after 35 days exposure. Taken together, this is consistent evidence that CA activity is a sensitive biochemical response in corals to Cu exposure. Interestingly, CA activity has also been identified as a key biochemical response in organisms for the detection of metal toxic effects in estuaries (Monserrat et al., 2007). From a mechanistic point of view, Cu has been shown to act as a non-competitive inhibitor of CA (Kaya et al., 2013), thereby changing the overall shape of the enzyme site for the normal substrate, slowing or preventing the reaction taking place (Cleland, 1963).

Zooxanthellae are located in the endodermal cells of corals tissue, a site of difficult access to DIC from seawater to be used in photosynthesis. CO_2 diffuses through coral cells and is converted into HCO_3^- by CA, thus avoiding diffuse loss of CO_2 (Bertucci et al., 2013). In order to provide DIC to zooxanthellae, CA associated with the membrane of zooxanthellae promotes the conversion of HCO_3^- into CO_2, which is absorbed and fixed by microalgae (Leggat et al., 2002; Bertucci et al., 2013). Therefore, a decrease in CA activity can affect photosynthesis by the reduction in the amount of CO_2 available to symbionts. Despite no effects in *FvFm* values were observed in the present study, it is not possible to assume that CA does not affect photochemical efficiency of zooxanthellae considering the short time period of exposure employed.

CA activity is also fundamental to the calcification process in corals. At the calcification site, CO_2 mainly from coral metabolism (Erez, 1978; Furla et al., 2000; Tambutté et al., 2011) undergoes a reversible hydration reaction catalyzed by CA followed by the carbonic acid (H_2CO_3) dissociation into HCO_3^- and H^+ (Bielmyer et al., 2010; Bertucci et al., 2013). Thus, the inhibitory effect of Cu on CA activity may decrease the amount of available substrate (HCO_3^-) for use in calcification and growth of the skeleton. Also, CA is also related to acid-base balance.

Biochemical responses to increasing Cu concentrations observed in *M. harttii* using two different experimental systems - controlled laboratory conditions and marine mesocosm - were very similar. However, the effects of Cu exposure on CA activity were more prominent at mesocosm conditions, even though Cu concentrations tested in laboratory conditions were more than 2 times higher compared to those tested in the mesocosm system. Based on the present findings, both experimental setups are valid and useful to evaluate metals toxicity in corals. Under controlled experimental laboratory conditions one factor can be tested at a time, allowing further understanding on toxicity mechanisms (Tremblay et al., 2010). On the other hand, using mesocosm systems for this purpose is also an efficient approach since they enable to integrate the complexity of natural environment parameters that are very difficult to achieve in laboratory conditions (Kangas and Adey, 1996). Despite the fact we are not able to specifically point out the reasons, it is very likely that the differences observed between the two experimental set ups regarding Cu toxicity and basal levels of enzymes activity (not statistically significant) are related to a more pronounced daily variation in environmental parameters (e.g. light incidence, dissolved nutrients, etc) in the mesocosm system, as well as to differences in water motion and renewal used in the two experiments. For instance, dissolved materials involved in coral metabolism can be limited by diffusion in low water motion environments (Jokiel, 1978; Reidenbach et al., 2006). Therefore corals in the mesocosm system would experience enhanced conditions of exchanging substances across the interface between the seawater and their tissues compared to the laboratory closed-system condition.

Finally, most dissolved Cu concentrations tested in the present study are close to concentrations of dissolved Cu allowed by South and North American environmental agencies (CONAMA, 2005 and EPA, 2005, respectively). Those Cu concentrations are likely found in coral reefs worldwide, with studies on reef polluted areas recording higher concentrations reaching up to 30 μg Cu/L (e.g. Jones, 2010). Cu contamination can ultimately cause ecological and economic disservice associated with the loss of integrity of coral reefs. Further, in the context of an era of climate change, the toxicity of metals is expected to be enhanced due to increasing seawater temperature and acidification, resulting in even more severe impacts to coral reefs (Negri and Hoogenboom, 2011; Fonseca et al., 2017; Banc-Prandi and Fine, 2019). Faced with the increasing threats to coral reefs, there is growing interest in approaches that evaluates exposure and effects of environmental chemicals, such as metals, by the use of biomarkers. We expect the present findings can contribute for the development of biomarker-based environmental monitoring programs in coral reefs.

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